Supplementary Information

Plasmids

Vegfaa-121 R121A and Vegfaa-165 R165A were generated by PCR using mismatched primers listed in supplemental Table 1. To generate Vegfaa-121 and -165 Myc-tag, which correspond to zebrafish Vegfaa with a 10-residue c-Myc epitope inserted between the signal peptide and the knot motif, we first introduced a BamHI restriction site by site directed mutagenesis using primers listed in supplemental Table 1. This plasmid was then used as a template to introduce the Myc-tag sequence. Oligos encoding the Myc-Tag sequence and listed in supplemental Table 1 were annealed in a thermo block at 90–95 °C for 5 minutes followed by a slow cooling to room temperature (on the bench) and cloned into pcDNA 3.1 between BamHI sites. To generate the Myc-tagged Pgfb, a c-Myc epitope was inserted between the signal peptide and the knot motif by site directed mutagenesis using the primers listed in supplemental Table 1. Signal peptide cleavage sites were identified using the SignalP 4.1 software (http://www.cbs.dtu.dk/services/SignalP/). Plasmids were linearized with SmaI at room temperature for 3 hours. All constructs were verified by sequencing. Capped RNA was synthesized using mMESSAGE mMACHINE T7 kit (Life Technologies, Carlsbad, CA) and purified using RNeasy Mini kit (Zymo research, Irvine, CA).

Microinjection of morpholinos

vegfaa (5' - GTATCAAATAAACAACCAAGTTCAT - 3') morpholino was purchased from GeneTools LLC (Philomath, OR) and injected at 2 ng per embryo as previously reported.[10]

Wholemount In Situ Hybridization

Wholemount in situ hybridization was performed as previously described using probes for *flt4* and *efnb2a*.[10]

Cell culture and transfection

HEK293 (Human Embryonic Kidney 293) or COS-1 cell cultures were maintained at 37 °C in 5% CO2, 95% air in appropriate medium containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were transfected with cDNAs in antibiotic-free medium 12-24 h before experiments, using FuGene HD (Roche, Basel, Switzerland) at a 3:1 ratio (μl:μg nucleic acid) and 0.18 μg DNA per cm^2.

Immunofluorescence

COS-1 and HEK293 cells were transfected with Myc-tagged Vegfaa isoforms and VEGFR2 and fixed with 4% paraformaldehyde and incubated for 20 min in blocking buffer (PBS containing 6 mm glucose, 1 mM pyruvate, 1% bovine serum albumin and 2% goat serum) and then for 20 min in 70 μg/ml mouse anti-Myc (Santa Cruz) antibody in blocking buffer. Cells were then rinsed and incubated another 20 min with 10 μg/ml goat anti-mouse IgG-conjugated Alexa Fluor 488 in blocking buffer. After incubation with secondary antibodies, cells were rinsed extensively in PBS, and cover glasses were mounted with VectaMount (Vector Laboratories, Burlingame, CA) containing 4′,6-diamidino-2-phenylindole to stain nuclei.

Genome editing

For the CRISPR work, oligos listed in supplemental Table 1 were annealed in a thermo block at 90–95 °C for 5 minutes followed by slow cooling to room temperature (on the bench) and cloned in gRNA plasmid between BsmBI sites. For making nls-zCas9-nls RNA, the template DNA was linearized by NotI (pCS2-nls-zCas9-nls) or XbaI (pT3TS-nls-zCas9-nls) digestion and purified using a QIAprep column (Qiagen, Venlo, Germany). Capped nls-zCas9-nls RNA was synthesized using mMESSAGE mMACHINE SP6 or T3 kit (Life Technologies, Carlsbad, CA) and purified using RNeasy Mini kit (Zymo research, Irvine, CA). For making gRNA, the template DNA was linearized by BamHI digestion and purified using a QIAprep column. gRNA was generated by in vitro transcription using T7 RNA polymerase MEGA short script T7 kit (Life Technologies). After in vitro transcription, the gRNA (approx 100 nucleotides long) was purified using RNA clean and concentrator (Zymo research, Irvine, CA). All constructs were verified by sequencing. 300 pg of CAS9 mRNA and 50 pg of gRNA were co-injected in the cell at the one cell stage.

Generation of *flt1*^{fh390} zebrafish mutant

flt1^{fh390} zebrafish mutant was identified in a chemical mutagenesis screen using a MiSeq device (Illumina).[19] A C to T substitution in *flt1* exon 15 (TTATACCTGT[C|T]AAGCCACCAA) leads to a premature stop codon Q721*.

Genotyping

Embryos or fin-clips were placed in PCR tubes, with 50 µl elution buffer (10 mM Tris-Cl, pH 8.5) and 1 mg/ml Proteinase K added to each well and then incubated at 55° C for 2 hrs. The samples were then heated to 95° C for 10 min to inactivate Proteinase K. Primers were designed using primer3: http://biotools.umassmed.edu/bioapps/primer3_www.cgi.
vegfaa^{bns1} *and flt1*^{fh390} animals were genotyped by HRMA whereas *vegfab*^{bns92} animals were genotyped by PCR using primers listed in supplemental Table 1.
Eco Real-Time PCR System (Illumina, San Diego, CA) was used for PCR reaction and high-resolution melt analysis. DyNAmo SYBR green (Thermo Fisher Scientific, Waltham, MA) was used in these experiments. PCR reaction protocol was 95° C for 15 sec, then 40 cycles of 95° C for 2 sec, 60° C for 2 sec, and 72° C for 2 sec. After PCR, the reaction was heated to 95° C for 10 sec, then cooled to 4° C. Following the PCR, a high resolution melting curve was generated by collecting SYBR-green fluorescence data in the 65-95°C range. The temperature was set to increase at 0.1° C /second. Eco software for melting curve analysis is based on normalization and display of derivative plots. The analyses were performed on normalized derivative plots.

Supplementary Figures:

Figure S1: *vegfaa*^{bns1} mutants lack intersegmental vessels.

(A) Confocal micrographs of 26 hpf *TgBAC(etv2:EGFP)* WT siblings and *vegfaa* mutant embryos in lateral views. Asterisks denote lack of ISVs.
(B) Wholemount *in situ* hybridization of *flt4* expression in 26 hpf embryos in lateral views. Arrows point to *flt4* positive cells. Scale bars, 100 µm.

Figure S2: Generation of *vegfab*^{bns92} mutant.
(A) A gRNA was designed to target exon 2 of *vegfab*. (B) Sequence alignment of the CRISPR generated allele. *vegfab*^{bns92} allele has a seven nucleotide insertion leading to a premature stop codon. (C) Cartoon representation of WT Vegfab isoforms -171, -210 and Vegfab^{bns92}. Vegfab-171 and -210 consists of a signal peptide, a knot motif, and a heparin and Nrp1 binding domain. *vegfab*^{bns92} encodes a truncated polypeptide (p.Met88Trpfs*18).

Figure S3: *vegfab* mutants exhibit minor angiogenesis defects.
(A) Top, brightfield micrograph of a *vegfab*^{bns92} mutant in lateral view. Bottom, confocal micrograph of a 48 hpf *TgBAC(etv2:EGFP) vegfab*^{bns92} mutant in lateral view. Asterisks denote the occasional lack of ISVs. (B) Left, brightfield micrograph of a 48 hpf *vegfab* mutant in dorsal view. Right, confocal micrograph of a 48 hpf *TgBAC(etv2:EGFP) vegfab* mutant in dorsal view. Arrows point to PHBC, BA and CtAs. Asterisks indicate impaired CtA formation. Scale bars, 100 μm.

Figure S4: **Determinants of Vegfaa binding to Vegfr2 and Nrp1 *in vivo*. A**) Top, epifluorescence images show HEK293 cells transiently expressing engineered Myc-tag Vegfaa-121 or Vegfaa-165, wildtype (top) or K84A (bottom) and labeled with anti-Myc (green) antibody. Bottom, schematic representation of Vegfaa binding to Vegfr2.
(B) Top, brightfield micrograph of 48 hpf *vegfaa* mutant embryos injected with *vegfaa-121* K84A *or -165* Y47/K84A in lateral view. Arrow points to pericardial edema. Bottom, confocal micrograph of 48 hpf *TgBAC(etv2:EGFP) vegfaa* mutant embryos injected with *vegfaa-121* K84A *or -165* Y47/K84A in lateral view. Asterisks indicate lack of ISVs and axial vessel. (C) Top, brightfield micrograph of 48 hpf *vegfaa*$^{bns1/bns1}$ embryo injected with *vegfaa-165* R165A in lateral view. Arrow points to lack of pericardial edema in the injected embryo. Bottom, confocal micrograph of 48 hpf *TgBAC(etv2:EGFP) vegfaa* mutant embryo injected with *vegfaa-165 R165A* in lateral view. Arrow points to luminized blood vessel in the DA region.

Figure S5: Pgfb rescued *vegfaa* mutants through Vegfr1 signaling.
(A) Schematic of *flt1*^{fh390} mutant protein. WT Flt1 consists of both a membrane-bound and a soluble isoform (top). The *flt1*^{fh390} mutation affects the membrane-bound isoform leading to a lack of both the transmembrane and tyrosine kinase domains without affecting the soluble isoform.
(B, C) Experimental design. Embryos deriving from *vegfaa*$^{bns1/+}$;*flt1*$^{fh390/+}$ incrosses were injected with 200 pg of *pgfb* mRNA at the one-cell stage.
(D) Quantification of rescue experiment. 25% of the progeny from double heterozygous *vegfaa*;*flt1* animals exhibit a *vegfaa* mutant phenotype (32/129) at 48 hpf while injection of *pgfb* mRNA reduced the phenotype to 12,9 % of the clutch (24/185).
(E) High-resolution melt analysis genotyping of the 24 embryos showing a vascular phenotype. Embryos were *vegfaa*$^{bns1/bns1}$;*flt1*$^{fh390/fh390}$ (n=11/24), *vegfaa*$^{bns1/bns1}$;*flt1*$^{fh390/+}$ (n=10/24) or *vegfaa*$^{bns1/bns1}$;*flt1*$^{+/+}$ (n=3/24), revealing an impaired rescue ability of *pgfb* depending on Flt1 function (P value represents chi-square test for mendelian ratios $P<0.05$).

Figure S6: Pgfb D63A-E64A-E67A poorly rescues *vegfaa* mutants.
(A) Mutagenesis of Vegfaa-165 and Pgfb for three conserved amino-acids mediating Vegfr1 binding. Arrows point to D63, E64 and E67 respectively.
(B, C) Embryos deriving from an incross of *vegfaa* mutants were injected with *vegfaa-165* or *pgfb D63A-E64A-E67A* mRNA.
(D) Quantification of rescue experiment using *vegfaa-165, vegfaa-165 D63A-E64A-E67A, pgfb* and *pgfb D63A-E64A-E67A* mRNA. Mutations moderately decreased the Vegfaa-165 rescue efficiency from 100% to around 73% whereas Pgfb rescue efficiency drastically decreased from 100% to around 43%, indicating that Pgfb mediates its rescue effects through Vegfr1. Note that for each sample, the rescue efficiency was normalized to the wild-type isoform which was set at 100%.

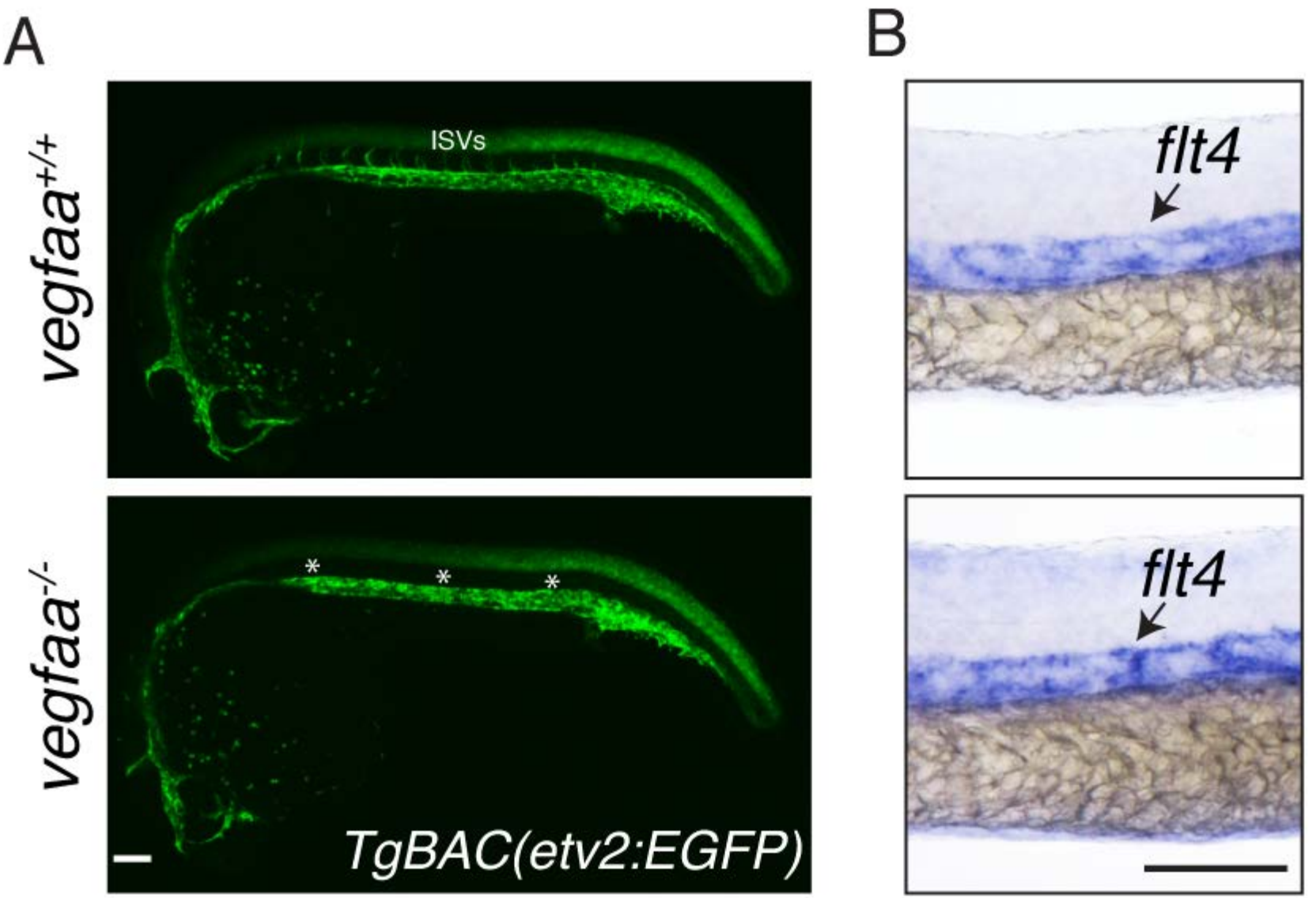
Figure S7: Vegfaa dominant negative molecules block ISV sprouting.
(A, B) Left, brightfield micrograph of 26 hpf WT or *vegfaa-121 E64A* mRNA injected embryos in lateral view. Right, confocal micrograph of 26 hpf *TgBAC(etv2:EGFP)* WT or *vegfaa-121 E64A* mRNA injected embryos in lateral view. Asterisks indicate lack of ISVs. Scale bar, 100 μm.

Figure S8: Vegf dominant negative molecules block vascular development.
(A) Brightfield micrographs of 48 hpf WT embryos injected with indicated *vegfaa-121* mRNA in lateral views. Arrows point to pericardial edema. (B) Confocal micrographs of 48 hpf *TgBAC(etv2:EGFP)* WT embryos injected with indicated *vegfaa* mRNA in lateral views. Asterisks indicate lack of ISVs. Scale bar, 100 μm.

Figure S9: Vegfaa dominant negative molecules exacerbate the vascular phenotypes of *vegfaa;vegfab* double mutants.
(A) Brightfield micrograph of 48 hpf *vegfaa;vegfab* double mutants uninjected or injected with *vegfaa-121 E64* mRNA in lateral view. Arrows point to pericardial edema.
(B) Confocal micrograph of 48 hpf *TgBAC(etv2:EGFP) vegfaa;vegfab* double mutants uninjected or injected with *vegfaa-121 E64* mRNA in lateral view. Asterisks indicate reduced ISV sprouting and brain vasculature in the injected embryo. Scale bar, 100 μm.

Figure S10: Vegfaa dominant negative molecules block brain and intestinal angiogenesis.
(A) Confocal micrographs of 72 hpf *TgBAC(etv2:EGFP)* WT, *vegfaa* mutant or *vegfaa-121 E64A* or *K84A* mRNA injected larvae in lateral views. Asterisks indicate lack of brain vasculature in the injected larvae. (B) Brightfield micrographs of 48 hpf WT, *vegfaa* mutant or *vegfaa-121 E64A* or *K84A* mRNA injected larvae in lateral views. Arrows point to pericardial edema. (C) Confocal micrographs of 72 hpf *TgBAC(etv2:EGFP)* WT, *vegfaa* mutant or *vegfaa-121 E64A* or *K84A* mRNA injected larvae in lateral views. Asterisks indicate lack of ISVs, ICVs and SIV in the injected embryos. Scale bars, 100 μm.

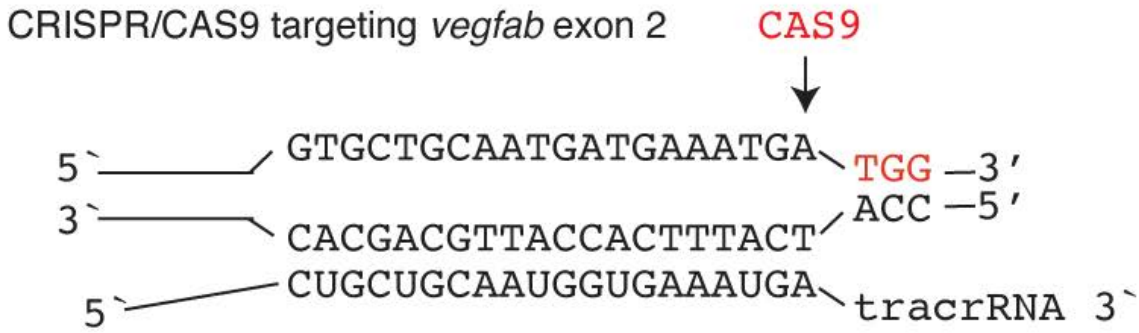
vegfaa^{bns1} mutant lack intersegmental vessels.



Rossi et al. Figure S1

Generation of *vegfab*^{bns92} mutant.

A



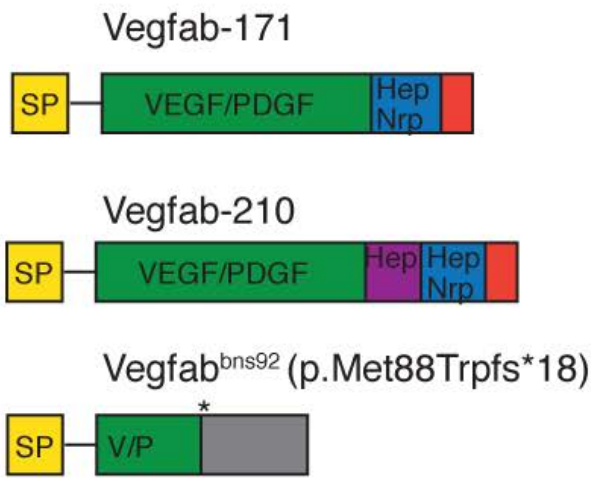
B

indel in *vegfab* zebrafish locus

wt CTGCAATGATGAAATG-------ATGGAATGCACCCCCA

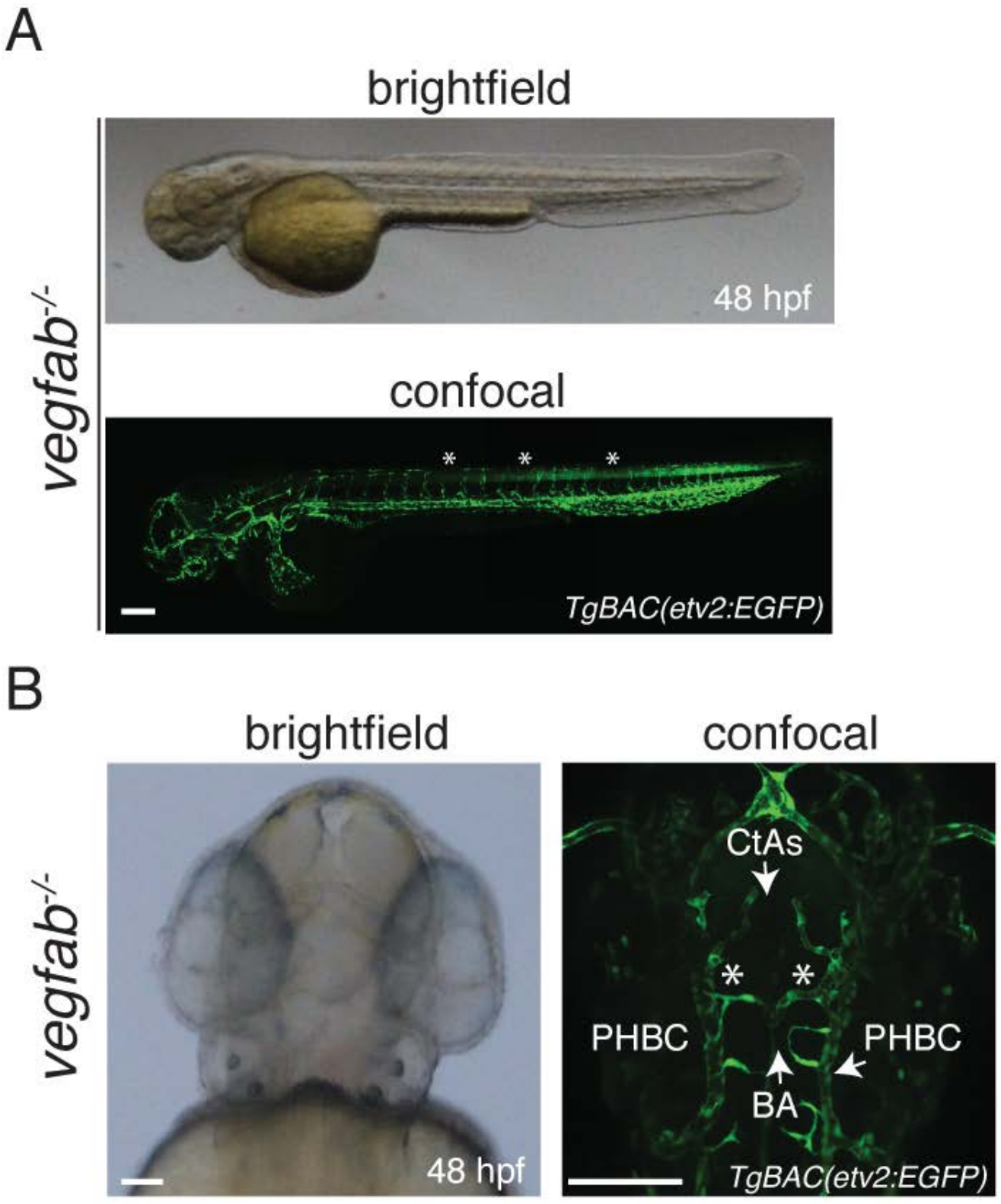
+7 CTGCAATGATGAAATGGAATGGAATGGAATGCACCCCCA

C



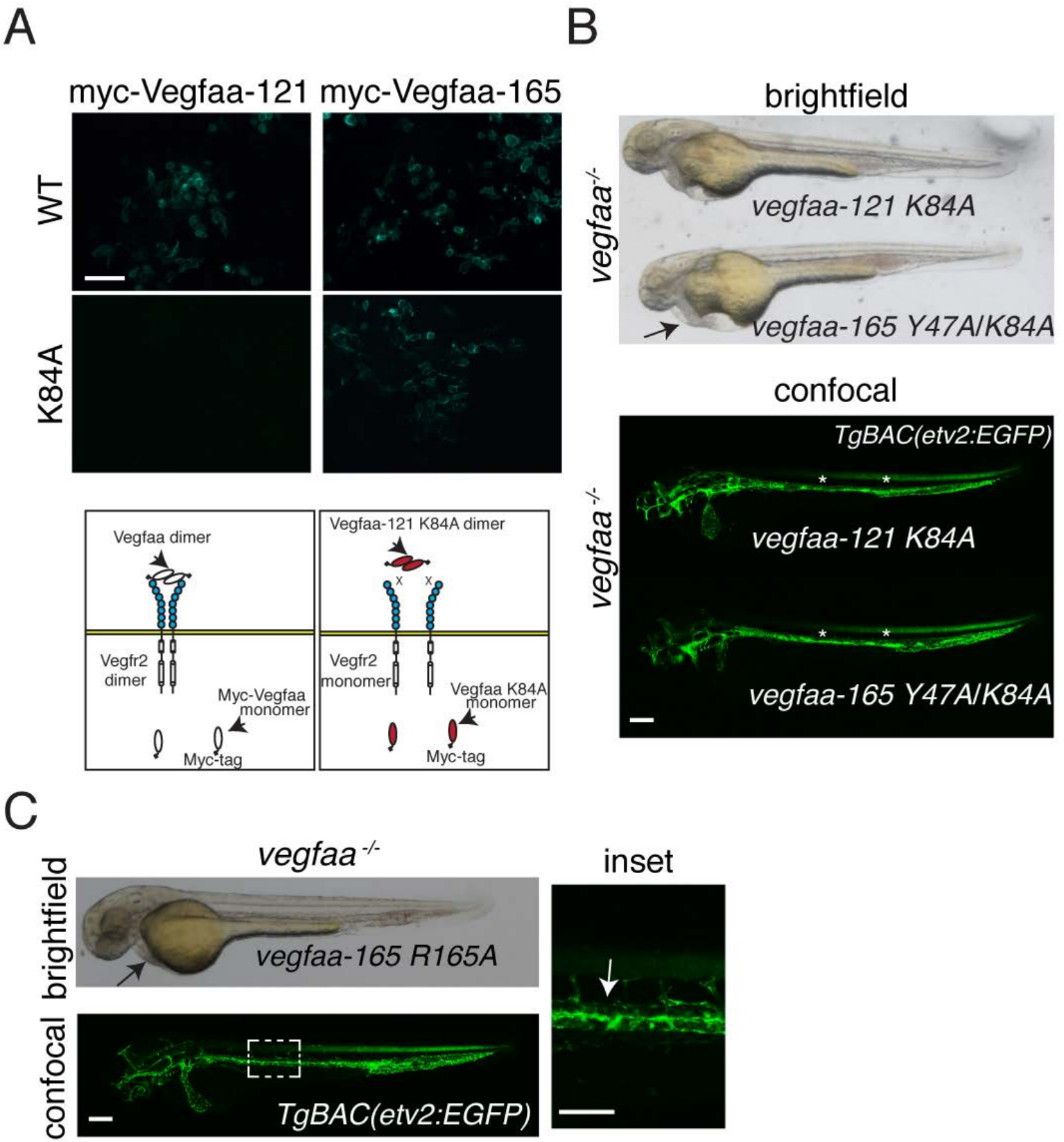
Rossi et al. Figure S2

vegfab^{bns92} mutants exhibit minor angiogenesis defects.



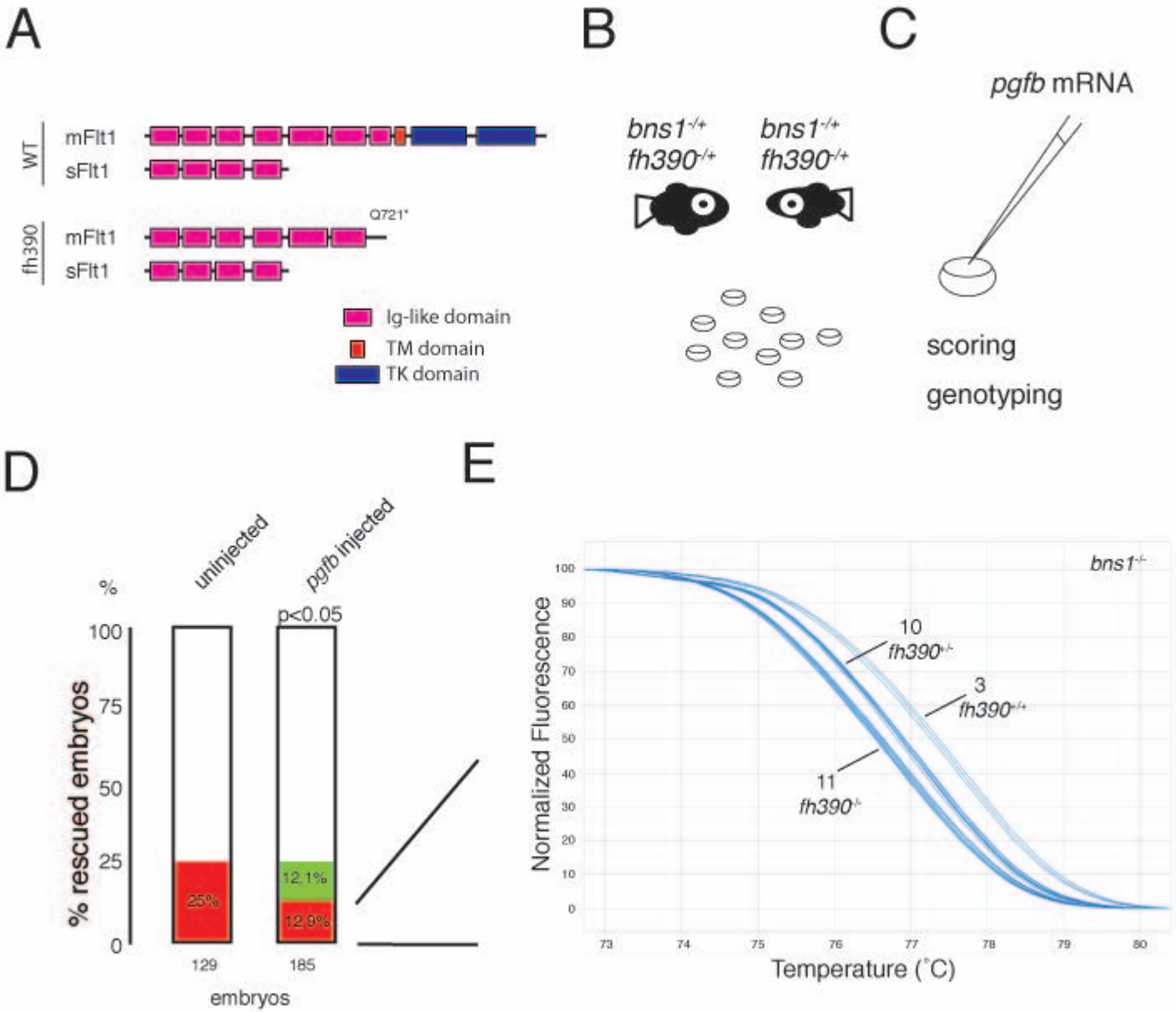
Rossi et al. Figure S3

Determinants of Vegfaa binding to Vegfr2 and Nrp1 *in vivo*.



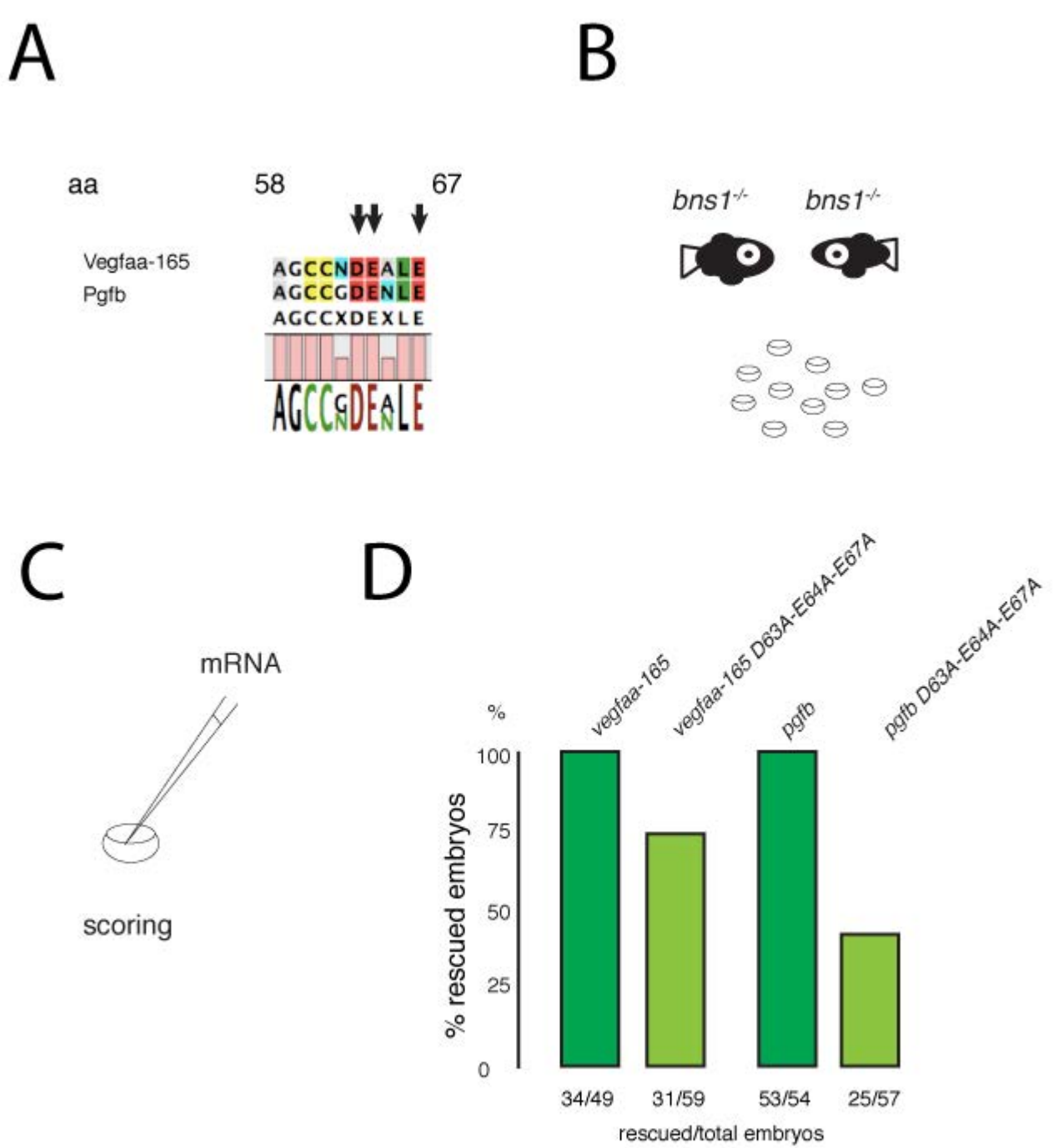
Rossi et al. Figure S4

Pgfb rescued *vegfaa* mutants through Vegfr1 signaling.



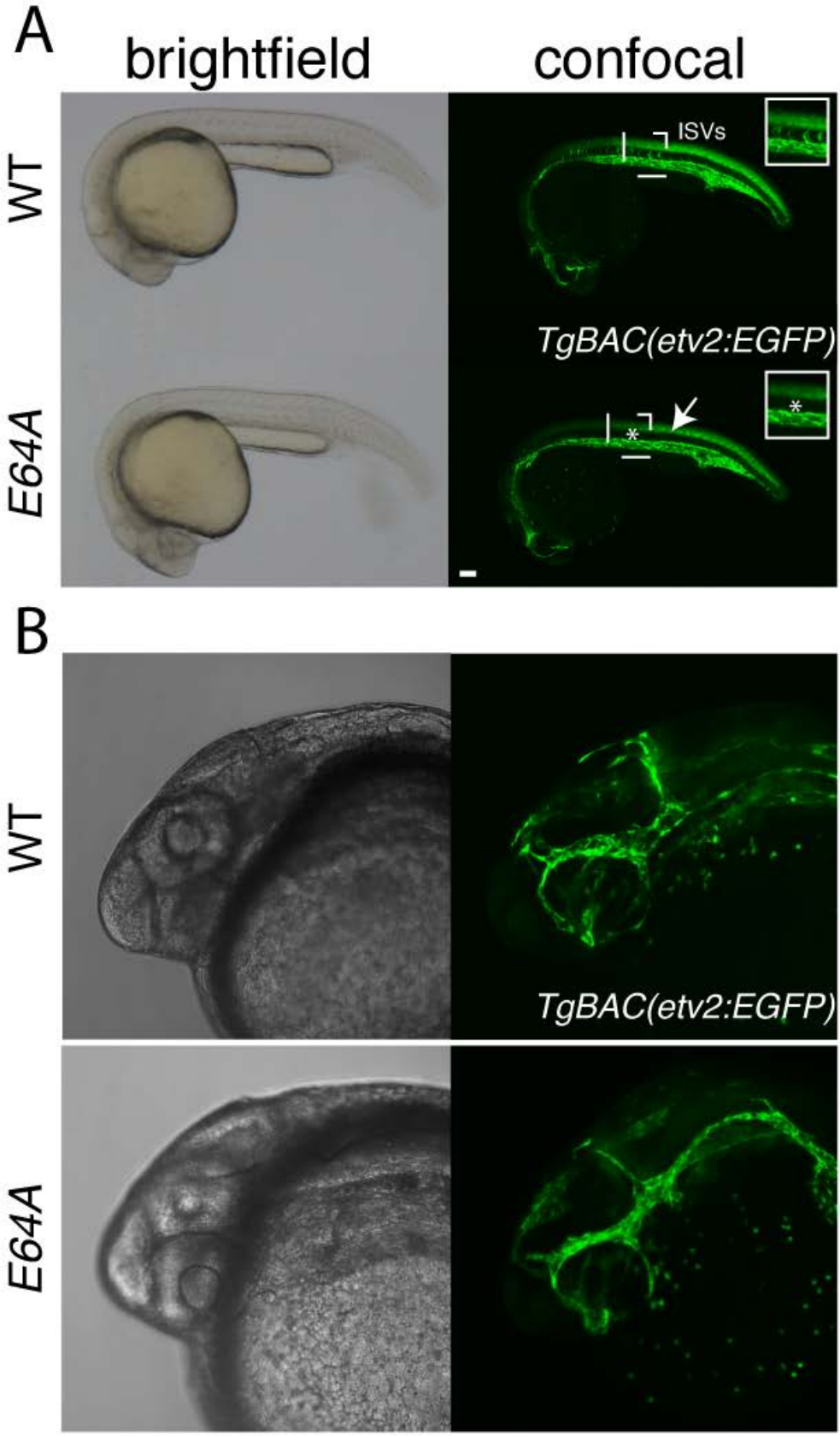
Rossi et al. Figure S5

Pgfb D63A-E64A-E67A poorly rescues *vegfaa* mutants.



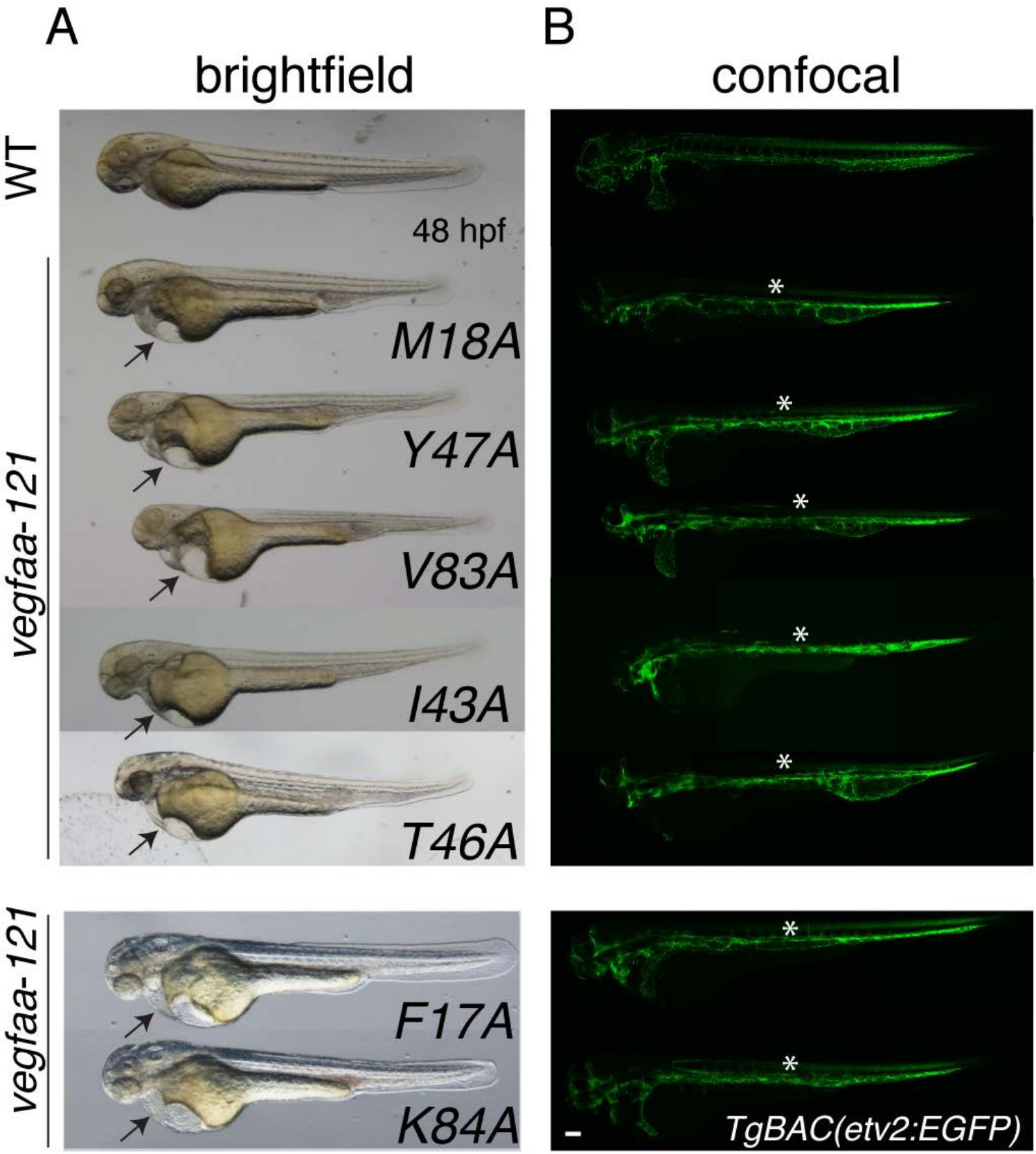
Rossi et al. Figure S6

Vegfaa dominant negative molecules block ISVs sprouting.



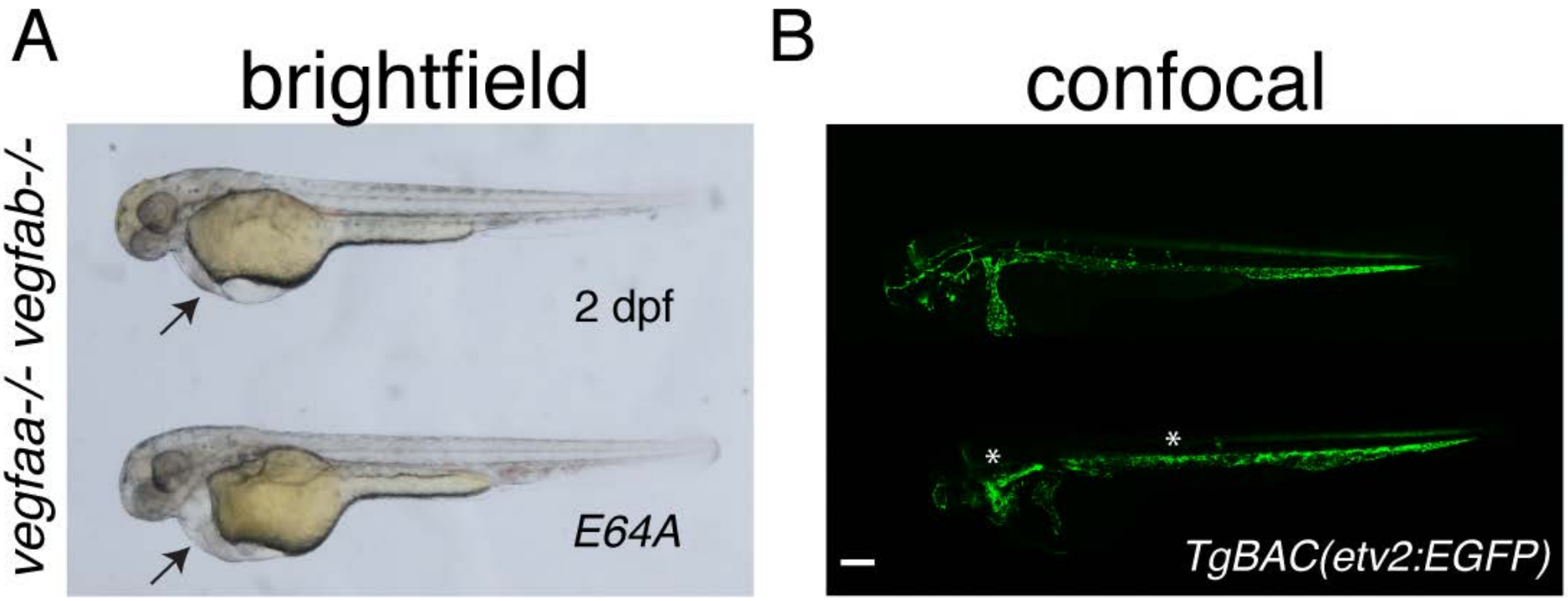
Rossi et al. Figure S7

Vegf dominant negative molecules block vascular development.



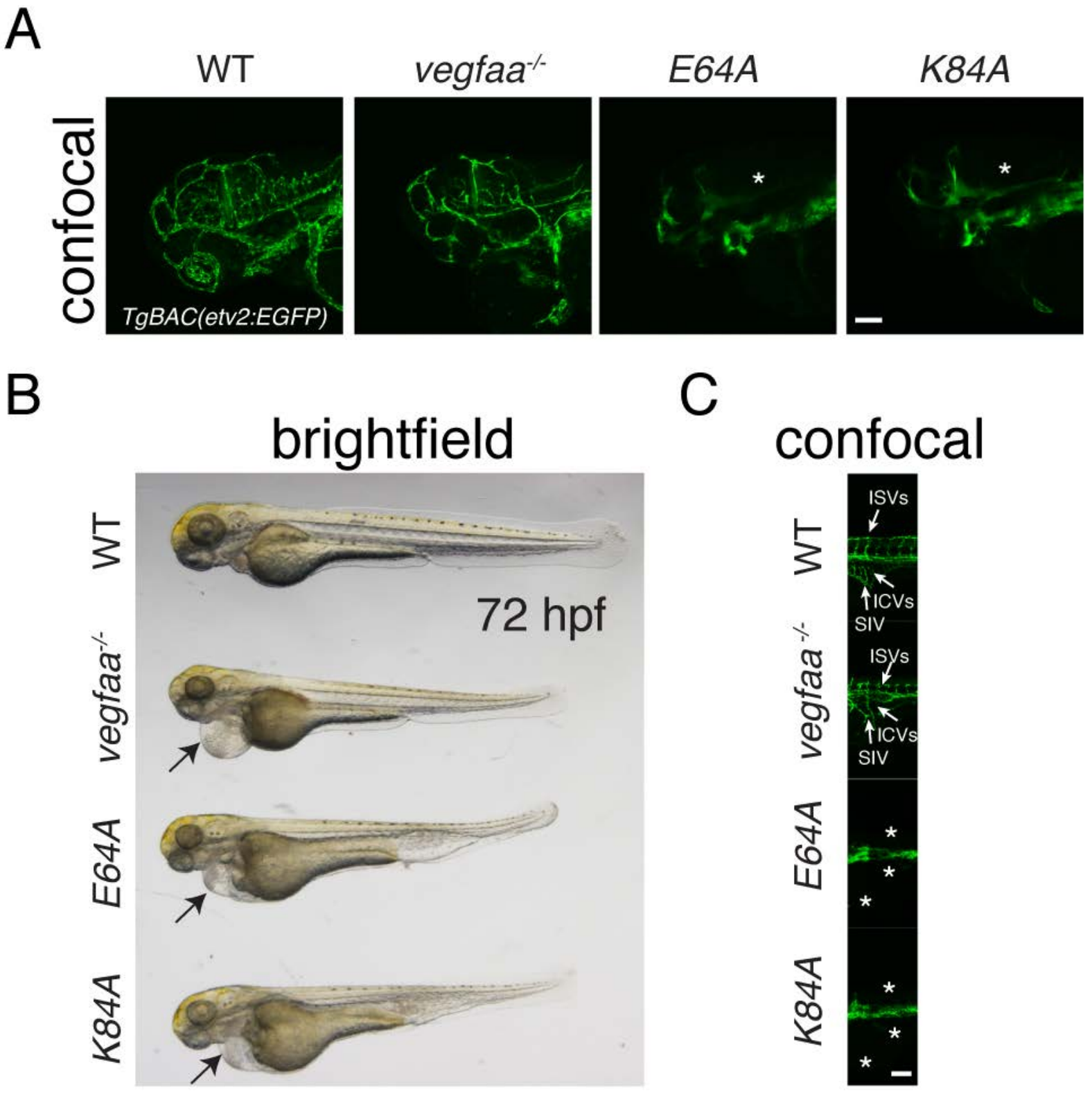
Rossi et al. Figure S8

Vegfaa dominant negative molecules exacerbate the vascular phenotype of *vegfaa*^{bns1};*vegfab*^{bns92} double mutants.



Rossi et al. Figure S9

Vegfaa dominant negative molecules block brain and intestinal angiogenesis.



Rossi et al. Figure S10